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Patentanmeldung Nr. Patent application No. Demande de brevet n°

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Page 2 de l'attestation

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Anti CD19xanti-CD3 bispecific single-chain polypeptides and uses thereof

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Novel CD19xCD3 specific polypeptides and uses thereof

The present invention relates to novel single-chain multifunctional polypeptides comprising at least two binding sites specific for the CD19 and CD3 antigen, respectively. The present invention further relates to a polypeptide, wherein the above-described polypeptide comprises at least one further domain, preferably of pre-determined function. Furthermore, the present invention relates to polynucleotides encoding said polypeptides as well as to vectors comprising said polynucleotides and to host cells transformed therewith and their use in the production of said polypeptides. In addition, the present invention relates to pharmaceutical and diagnostic compositions comprising any of the afore-described polypeptides, polynucleotides or vectors. A further object of the present invention is the use of the afore-mentioned polypeptides, polynucleotides and vectors for the preparation of pharmaceutical compositions for immunotherapy, preferably against B-cell malignancies such as non-Hodgkin lymphoma.

Despite the medical importance, research in B-cell mediated diseases such as non-Hodgkin lymphoma has produced only a small number of clinically usable data and conventional approaches to cure such diseases remain tedious and unpleasant and/or have a high risk of relapse. For example, although high dose chemotherapy as a primary treatment for high grade non-Hodgkin lymphoma may improve overall survival, about 50% of the patients still die of this disease (2-4). Moreover, low-grade non Hodgkin lymphoma like chronic lymphatic leukemia and mantle cell lymphoma are still incurable. This has stimulated the search for alternative strategies like immunotherapy. Antibodies directed against cell surface molecules defined by CD antigens represent a unique opportunity for the development of therapeutic reagents. The expression of certain CD antigens is highly restricted to specific lineage lymphohematopoietic cells and over the past several years, antibodies directed against lymphoid-specific antigens have been used to develop treatments that were

effective either in vitro or in animal models (5-13). In this respect CD19 has proved to be a very useful target. CD19 is expressed in the whole B lineage from the pro B cell to the mature B cell, it is not shed, is uniformly expressed on all lymphoma cells, and is absent from stem cells (8, 14). An interesting modality is the application of a bispecific antibody with one specificity for CD19 and the other for the CD3 antigen on T cells. However, bispecific antibodies thus far available suffer from low T-cell cytotoxicity and the need of costimulatory agents in order to display satisfactory biological activity.

Thus, the technical problem underlying the present invention was to provide means and methods useful for the treatment of B-cell mediated diseases such as various forms of non-Hodgkin lymphoma. The solution to said technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the present invention relates to a single-chain multi-functional polypeptide comprising

(a) a first domain comprising a binding-site of an immunoglobulin chain or an antibody specifically recognizing the CD19 antigen; and

(b) a second domain comprising a binding site of an immunoglobulin chain or an antibody specifically recognizing the CD3 antigen.

The terms "first domain" and "second domain" in accordance with the present invention mean that one binding site is directed against the pan B cell marker CD19, which is uniformly expressed on the vast majority of malignant B cells, the other binding site is directed against the CD3 antigen of human T cells.

The term "binding site" as used in accordance with the present invention denotes a domain comprising a three-dimensional structure capable of specifically binding to an epitope like native antibodies, free scFv fragments or one of their corresponding immunoglobulin chains, preferably the VH chain. Thus, said domain can comprise the VH and/or VL domain of an antibody or an immunoglobulin chain, preferably at least the VH domain. On the other hand, said binding sites contained in the polypeptide of the invention may comprise at least one complementarity determining region (CDR) of an antibody or immunoglobulin chain recognizing the CD19 and

CD3 antigen, respectively. In this respect, it is noted that the domains of the binding sites present in the polypeptide of the invention may not only be derived from antibodies but also from other CD19 or CD3 binding proteins, such as naturally occurring surface receptors or ligands. In accordance with the invention, said binding site is comprised in a domain.

The term "multifunctional polypeptide" as used herein denotes a polypeptide comprising at least two amino acid sequences derived from different origins, i.e. from two different molecules, optionally derived from different species wherein at least two of said origins specify the binding sites. Accordingly, said binding sites specify the functions or at least some functions of said multifunctional peptide. Such polypeptides include, for example, bispecific single-chain (bsc) antibodies.

The term "single-chain" as used in accordance with the present invention means that said first and second domain of the polypeptide are covalently linked, preferably in the form of a colinear amino acid sequence encodable by a nucleic acid molecule.

CD19 denotes an antigen that is expressed in the B lineage such as in the pro B cell and the mature B cell, it is not shed, is uniformly expressed on all lymphoma cells, and is absent from stem cells (8, 14).

CD3 denotes an antigen that is expressed on T-cells as part of the multimolecular T-cell receptor complex and that consists of three different chains CD3ε, CD3δ and CD3γ. Clustering of CD3 on T-cells, e.g. by immoblized anti-CD3-antibodies, leads to T-cell activation similar to the engagement of the T-cell receptor but independent from its clone typic specificity. Actually, most anti-CD3-antibodies recognize the CD3ε-chain.

Antibodies that specifically recognize CD19 or CD3 antigen are described in the prior art, e.g., in (24) and (25), respectively, and can be generated by conventional methods known in the art.

Bispecific CD19xCD3 antibodies retargeting T cell cytotoxicity on lymphoma cells in a MHC-independent manner have already been shown to be effective *in vitro* (5, 6, 9-11, 13), in animal models (7, 28) as well as in some pilot clinical trials (12, 29, 30). So far these antibodies were constructed by hybrid-hybridoma techniques or by covalently linking the monoclonal antibodies (31). More extensive clinical studies have been hampered by the fact that these antibodies have low biological activity

such that high dosages have to be applied and that application of the antibodies alone did not provide for a beneficial therapeutic effect. Furthermore, the availability of clinical grade material was limited.

Without being bound to a particular theory, it is believed that using the bispecific antibody-like format as defined above, thus generated polypeptides such as bispecific CD19xCD3 antibodies are usually capable of destroying CD19-positive target cells by recruitment of cytotoxic T-lymphocytes without any need for T-cell pre- and/or co-stimulation. This is in sharp contrast to all known bispecific CD19xCD3 antibodies produced according to other molecular formats and usually does not depend on the particular CD19- or CD3-antibody specificities used to construct, e.g., the bispecific single-chain antibody. The independence from T-cell pre- and/or co-stimulation may substantially contribute to the exceptionally high cytotoxicity mediated by the polypeptide of the invention as exemplified by the particular CD19xCD3 bispecific antibody described in the examples.

A further advantageous property of the polypeptide of the invention is that due to its small, relatively compact structure it is easy to produce and purify, thereby circumventing the problems of low yields, occurrence of ill-defined by-products, or laborious purification procedures (15-19) reported for CD19xCD3 specific antibodies hitherto produced from hybrid-hybridomas, by chemical linkage or by renaturation from bacterial inclusion bodies. In the following, the advantageous and unexpected properties of the polypeptide of the invention will be discussed in a non-limiting manner guided by the appended examples, including some of the preferred embodiments of the invention referred to hereinbelow, which illustrate the broad concept of the present invention.

In accordance with the present invention, a eukaryotic expression system was used that had been developed for the production of recombinant bispecific single chain antibodies (1) in order to generate a recombinant bispecific CD19xCD3 single chain antibody by expression in CHO cells. The fully functional antibody was easily purified from the culture supernatant by its C-terminal histidine tag on a Ni-NTA chromatography column. Specific binding to CD19 and CD3 was demonstrated by

FACS analysis. The resultant bscCD19xCD3 molecule of the invention showed some unexpected properties:

- it induced high lymphoma directed T cell cytotoxicity. Even at very low concentrations of 10-100 pg/ml and low E (effector):T(target) ratios of 5:1 and 2.5:1 significant specific lysis of lymphoma cell lines was observed. Compared to so far published CD19xCD3 antibodies produced by hybrid-hybridoma techniques which show cytotoxic activity in the range of several nanograms/ml, the bscCD19xCD3 antibody of the invention seems to be much more efficacious (5-7, 27).
- Even low concentrations of the bscCD19xCD3 of the invention were able to induce rapid lymphoma directed cytotoxicity (after 4 h) at low E:T ratios without the need of any T cell prestimulation. In contrast a conventional CD19xCD3 bispecific antibody (5-7, 27) showed no significant cytotoxic activity under these conditions (namely no T cell prestimulation, low E:T ratio) even at high concentrations up to 3000ng/ml. Although induction of cytotoxic activity without prestimulation has also been reported in the case of another conventional CD19xCD3 antibody this effect was achieved only at high concentrations and high E:T ratios (100 ng/ml, 27:1) (9) compared to the bscCD19xCD3 of the invention (100 pg/ml, 2.5:1). Moreover, a cytotoxic effect of this conventional antibody was observed only after 1 day of prestimulation with the bispecific antibody itself whereas the bscCD19xCD3 of the invention induced lymphoma-directed cytotoxicity already after 4 hours. To the knowledge of the inventors such rapid and specific cytotoxic activity of unstimulated T cells at such low concentrations and E:T ratios has not been described for other bispecific antibodies used so far. Although recently a anti-p185HER2/anti-CD3 bispecific $F(ab)_2$ antibody has been shown to induce cytotoxic activity at similar concentrations as the bscCD19xCD3 of the invention, this antibody required 24 hr prestimulation with IL-2 (32). Thus, the bscCD19xCD3 antibody of the invention reveals unique cytototoxic properties that discriminate this molecule from other bispecific antibodies that have been described.

The bscCD19xCD3 of the invention mediates cytotoxic effects that are antigen specific, demonstated by the facts

- that this antibody failed to lyse the plasmacytoma cell lines NCI and L363 which are cell lines of the B lineage not expressing the CD19 antigen; and
- that the cytotoxicity against lymphoma cells could be blocked by the parental anti-CD19 antibody HD37.

Blocking the perforin-pathway by calcium-deprivation with EGTA completely blocked bsc CD19xCD3-mediated cytoxicity suggesting that specific lysis is a T cell-mediated effect rather than a direct effect of the antibody itself.

Taken together, the bscCD19xCD3 antibody constructed according to general teaching of the invention is superior to so far described CD19xCD3 bispecific antibodies with respect to its considerably higher biological activity as well as the possibility of its fast and easy production, thereby yielding sufficient amounts of high quality clinical grade material

Therefore, the bscCD19xCD3 molecule of the invention and derivatives thereof are expected to be a suitable candidate to prove the therapeutic benefit of bispecific antibodies in the treatment of B-cell mediated diseases such as non-Hodgkin lymphoma in clinical trials.

In a preferred embodiment of the polypeptide of the invention said domains are connected by a polypeptide linker. Said linker is disposed between said first and said second domain, wherein said polypeptide linker preferably comprises plural, hydrophilic, peptide-bonded amino acids and connects the N-terminal end of said first domain and the C-terminal end of said second domain.

In a further preferred embodiment of the invention said first and/or second domain of the above-described polypeptide mimic a VH and VL region from a natural antibody. The antibody providing the binding site for the polypeptide of the invention can be, e.g., a monoclonal antibody, polyclonal antibody, chimeric antibody, humanized antibody, bispecific antibody, synthetic antibody, antibody fragment, such as Fab, Fv or scFv fragments etc., or a chemically modified derivative of any of these. Monoclonal antibodies can be prepared, for example, by the techniques as originally described in Köhler and Milstein, Nature 256 (1975), 495, and Galfré, Meth. Enzymol. 73 (1981), 3, which comprise the fusion of mouse myeloma cells to spleen

cells derived from immunized mammals with modifications developed by the art. Furthermore, antibodies or fragments thereof to the aforementioned antigens can be obtained by using methods which are described, e.g., in Harlow and Lane "Antibodies, A Laboratory Manual", CSH Press, Cold Spring Harbor, 1988. When derivatives of said antibodies are obtained by the phage display technique, surface plasmon resonance as employed in the BIAcore system can be used to increase the efficiency of phage antibodies which bind to an epitope of the CD19 or CD3 antigen (Schier, Human Antibodies Hybridomas 7 (1996), 97-105; Malmborg, J. Immunol. Methods 183 (1995), 7-13). The production of chimeric antibodies is described, for example, in WO89/09622. Methods for the production of humanized antibodies are described in, e.g., EP-A1 0 239 400 and WO90/07861. A further source of antibodies to be utilized in accordance with the present invention are so-called xenogenic antibodies. The general principle for the production of xenogenic antibodies such as human antibodies in mice is described in, e.g., WO 91/10741, WO 94/02602, WO 96/34096 and WO 96/33735.

Antibodies to be employed in accordance with the invention or their corresponding immunoglobulin chain(s) can be further modified using conventional techniques known in the art, for example, by using amino acid deletion(s), insertion(s), substitution(s), addition(s), and/or recombination(s) and/or any other modification(s) known in the art either alone or in combination. Methods for introducing such modifications in the DNA sequence underlying the amino acid sequence of an immunoglobulin chain are well known to the person skilled in the art; see, e.g., Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y.

In a preferred embodiment of the invention at least one of said domains in the above-described polypeptide is a single-chain fragment of the variable region of the antibody.

As well known, Fv, the minimum antibody fragment which contains a complete antigen recognition and binding site, consists of a dimer of one heavy and one light chain variable domain (V_H and V_L) in noncovalent association. In this configuration

that corresponds to the one found in native antibodies the three complementarity determining regions (CDRs) of each variable domain interact to define an antigen binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen binding specificity to the antibody. Frameworks (FRs) flanking the CDRs have a tertiary structure which is essentially conserved in native immunoglobulins of species as diverse as human and mouse. These FRs serve to hold the CDRs in their appropriate orientation. The constant domains are not required for binding function, but may aid in stabilizing V_H-V_L interaction. Even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although usually at a lower affinity than an entire binding site (Painter (1972) Biochem. 11:1327-1337). Hence, said domain of the binding site of the polypeptide of the invention can be a pair of V_H-V_L, V_H-V_H or V_L-V_L domains either of the same or of different immunoglobulins. The order of V_H and V_L domains within the polypeptide chain is not decisive for the present invention, the order of domains given herein above may be reversed usually without any loss of function. It is important, however, that the V_H and V_L domains are arranged so that the antigen binding site can properly fold.

In a preferred embodiment of the polypeptides of the invention said domains are arranged in the order V_L CD19 - V_HCD19 – V_HCD3 – V_LCD3.

As discussed above, said binding sites are preferably connected by a flexible linker, preferably by a polypeptide linker disposed between said domains, wherein said polypeptide linker comprises plural, hydrophilic, peptide-bonded amino acids of a length sufficient to span the distance between the C-terminal end of one of said domains comprising said binding sites and the N-terminal end of the other of said domains comprising said binding sites when the polypeptide of the invention assumes a conformation suitable for binding when disposed in aqueous solution. Preferably, said polypeptide linker comprises a plurality of glycine, alanine and/or serine residues. It is further preferred that said polypeptide linker comprises a plurality of consecutive copies of an amino acid sequence. Usually, the polypeptide linker comprises 1 to 15 amino acids although polypeptide linkers of more than 15

amino acids may work as well. In a preferred embodiment of the invention said polypeptide linker comprises 1 to 5 amino acid residues.

In a particularly preferred embodiment of the present invention said polypeptide linker in the polypeptide of the invention comprises 5 amino acids. As demonstrateed in the appended examples, said polypeptide linker advantageously comprises the amino acid sequence Gly Gly Gly Gly Ser.

In a particularly preferred embodiment, said first domain of the polypeptide of the invention comprises at least one CDR of the V_H and V_L region comprising the amino acid sequence encdoded by the DNA sequence depicted in Figure 8 from nucleotides 82 to 414 (V_L) and nucleotides 460-831 (V_H) and/or said second domain comprises at least one CDR of the V_H and V_L region comprising the amino acid sequence encoded by the DNA sequence depicted in Figure 8 from nucleotides 847 to 1203 (V_H) and nucleotides 1258 to 1575 (V_L). The CDRs contained in the variable regions depicted in Figure 8 can be determined, for example, according to Kabat, Sequences of Proteins of Immunological Interest (U.S. Department of Health and Human Services, third edition, 1983, fourth edition, 1987, fifth edition 1990). The person skilled in the art will readily appreciate that the binding site or at least one CDR derived therefrom can be used for the construction of a polypeptide of the invention. Preferably, said polypeptide comprises the amino acid sequence encoded by the DNA sequence as depicted in Figure 8 from nucleotides 82 to 1575. The person skilled in the art will readily appreciate that binding sites of the polypeptide of the invention can be constructed according to methods known in the art, e.g., as described in EP-A1 0 451 216 and EP-A1 0 549 581.

The domains of the binding sites of the polypeptide of the invention preferably have a specificity at least substantially identical to the binding specificity of the, e.g., antibody or immunoglobulin chain where they are derived from. Such binding site domains can have a binding affinity of at least $10^5 M^{-1}$, preferably not higher than $10^7 M^{-1}$ for the CD3 antigen and advantageously up to $10^{10} M^{-1}$ or higher for the CD19 antigen.

In a preferred embodiment of the polypeptide of the invention

(a) said binding site of the first domain has an affinity of at least about 10^{-7} M, preferably at least about 10^{-9} M and most preferably at least about 10^{-11} M; and/or

(b) said binding site of the second domain has an affinity of less than about 10^{-7} M, preferably less than about 10^{-6} M and most preferably in order of 10^{-5}M.

In accordance with the preferred embodiments referred to above, it is advantageous if the binding site recognizing the CD19 antigen has a high affinity in order to capture the target cells to be destroyed with high efficiency. On the other hand, the binding affinity of the binding site recognizing the CD3 antigen should be in the order of those of the natural CD3 receptor or of that usually found for the interaction of the T-cell receptor with its ligand, that is an MHC-peptide complex on the target cell surface.

In a preferred embodiment of the invention, the polypeptide described above is a bispecific single-chain antibody.

The present invention further relates to a polypeptide comprising at least one further domain, said domains being linked by covalent or non-covalent bonds.

The linkage can be based on genetic fusion according to the methods known in the art and described above or can be performed by, e.g., chemical cross-linking as described in, e.g., WO 94/04686. The additional domain present in the polypeptide of the invention may preferably be linked by a flexible linker, advantageously a polypeptide linker to one of the binding site domains wherein said polypeptide linker comprises plural, hydrophilic, peptide-bonded amino acids of a length sufficient to span the distance between the C-terminal end of one of said domains and the N-terminal end of the other of said domains when said polypeptide assumes a conformation suitable for binding when disposed in aqueous solution. Preferably, said polypeptide linker is a polypeptide linker as described in the embodiments hereinbefore. The polypeptide of the invention may further comprise a cleavable linker or cleavage site for proteinases, such as enterokinase; see also the appended examples.

Furthermore, said additional domain may be of a predefined specificity or function. For example, the literature contains a host of references to the concept of targeting bioactive substances such as drugs, toxins, and enzymes to specific points in the

body to destroy or locate malignant cells or to induce a localized drug or enzymatic effect. It has been proposed to achieve this effect by conjugating the bioactive substance to monoclonal antibodies (see, e.g., N.Y. Oxford University Press; and Ghose, (1978) J. Natl. Cancer Inst. 61:657-676).

In this context, it is also understood that the polypeptides according to the invention may be further modified by conventional methods known in the art. This allows for the construction of chimeric proteins comprising the polypeptide of the invention and other functional amino acid sequences, e.g., nuclear localization signals, transactivating domains, DNA-binding domains, hormone-binding domains, protein tags (GST, GFP, h-myc peptide, FLAG, HA peptide) which may be derived from heterologous proteins. As described in the appended examples, the polypeptide of the invention preferably comprises a FLAG-tag of about 8 amino acids in length; see Figure 8.

The polypeptides of the invention can be used therapeutically in patients having a B-cell related autoimmune disease such as myasthenia gravis, Morbus Basedow, Hashimoto thyreoiditis, or Goodgasture syndrome. Such therapy can be accomplished by, for example, the administration of polypeptides of the invention. Such administration can utilize unlabeled as well as labeled polypeptides.

For example, the polypeptides of the invention could be administered labeled with a therapeutic agent. These agents can be coupled either directly or indirectly to the antibodies or antigens of the invention. One example of indirect coupling is by use of a spacer moiety. These spacer moieties, in turn, can be either insoluble or soluble (Diener, Science, 231:148, 1986) and can be selected to enable drug release from the antigen at the target site. Examples of therapeutic agents which can be coupled to the polypeptides of the invention for immunotherapy are drugs, radioisotopes, lectins, and toxins. The drugs which can be conjugated to the polypeptides of the invention include compounds which are classically referred to as drugs such as mitomycin C, daunorubicin, and vinblastine.

In using radioisotopically conjugated polypeptides of the invention for, e.g., immunotherapy, certain isotopes may be more preferable than others depending on such factors as leukocyte distribution as well as stability and emission. Depending on the autoimmune response, some emitters may be preferable to others. In general, α

and β particle-emitting radioisotopes are preferred in immunotherapy. Preferred are short range, high energy α emitters such as ^{212}Bi. Examples of radioisotopes which can be bound to the polypeptides of the invention for therapeutic purposes are ^{125}I, ^{131}I, ^{90}Y, ^{67}Cu, ^{212}Bi, ^{212}At, ^{211}Pb, ^{47}Sc, ^{109}Pd and ^{188}Re.

Lectins are proteins, usually isolated from plant material, which bind to specific sugar moieties. Many lectins are also able to agglutinate cells and stimulate lymphocytes. However, ricin is a toxic lectin which has been used immunotherapeutically. This is accomplished by binding the α-peptide chain of ricin, which is responsible for toxicity, to the polypeptide to enable site specific delivery of the toxic effect.

Toxins are poisonous substances produced by plants, animals, or microorganisms that, in sufficient dose, are often lethal. Diphtheria toxin is a substance produced by Corynebacterium diphtheria which can be used therapeutically. This toxin consists of an α and β subunit which under proper conditions can be separated. The toxic A component can be bound to a polypeptide of the invention and be used for site specific delivery to the interacting B-cell and T-cell which have brought into close proximity via binding to the polypeptide of the invention.

Other therapeutic agents such as described above which can be coupled to the polypeptide of the invention, as well as correspoding ex vivo and in vivo therapeutic protocols, are known, or can be easily ascertained, by those of ordinary skill in the art. Wherever appropriate the person skilled in the art may use a polynucleotide of the invention described hereinbelow encoding any one of the above described polypeptides or the corresponding vectors instead of the proteinaeous material itself.

Thus, the person skilled in the art will readily appreciate that the polypeptide of the invention can be used for the construction of other polypeptides of desired specificity and biological function. The polypeptides of the invention are expected to play an important therapeutic and scientific role in particular in the medical field, for example, in the development of new treatment approaches for B-cell related disorders such as certain forms of cancer and autoimmune diseases or as interesting tools for the analysis and modulation of the corresponding cellular signal transduction pathways.

In a preferred embodiment of the invention, said at least one further domain comprises a molecule selected from the group consisting of effector molecules

having a conformation suitable for biological activity, amino acid sequences capable of sequestering an ion, and amino acid sequences capable of selective binding to a solid support or to a preselected antigen.

Preferably, said further domain comprises an enzyme, toxin, receptor, binding site, biosynthetic antibody binding site, growth factor, cell-differentiation factor, lymphokine, cytokine, hormone, a remotely detectable moiety, anti-metabolite, a radioactive atom or an antigen. Said antigen can be, e.g., tumor antigen, a viral antigen, a microbial antigen, an allergen, an auto-antigen, a virus, a microorganism, a polypeptide, a peptide or a plurality of tumor cells.

Furthermore, said sequence capable of sequestering an ion is preferably selected from calmodulin, methallothionein, a fragment thereof, or an amino acid sequence rich in at least one of glutamic acid, aspartic acid, lysine, and arginine.

In addition, said polypeptide sequence capable of selective binding to a solid support can be a positively or negatively charged amino acid sequence, a cysteine-containing amino acid sequence, avidin, streptavidin, a fragment of Staphylococcus protein A, GST, a His-tag, a FLAG-tag or Lex A. As is described in the appended Examples, the polypeptide of the invention exemplified by a single-chain antibody has also been expressed with an N-terminal FLAG-tag and/or C-terminal His-tag that allow for easy purification and detection. The FLAG-tag used in the example comprises 8 amino acids (see Figure 8) and is thus preferably used in accordance with the present invention. However, FLAG-tags comprised of shortened versions of the FLAG used in the appended example such as the amino acid sequence Asp-Tyr-Lys-Asp are suitable as well.

The effector molecules and amino acid sequences described above may be present in a proform which itself is either active or not and which may be removed, when, e.g., entering a certain cellular environment.

In a most preferred embodiment of the invention, said receptor is a costimulatory surface molecule important for T-cell activation or comprises an epitope binding site or a hormone binding site.

In a further most preferred embodiment of the invention, said costimulatory surface molecule is CD80 (B7-1) or CD86 (B7-2).

Yet in a further embodiment, the present invention relates to polynucleotides which upon expression encode the above-described polypeptides. Said polynucleotides may be fused to suitable expression control sequences known in the art to ensure proper transcription and translation of the polypeptide.

Said polynucleotide may be, e.g., DNA, cDNA, RNA or synthetically produced DNA or RNA or a recombinantly produced chimeric nucleic acid molecule comprising any of those polynucleotides either alone or in combination. Preferably said polynucleotide is part of a vector. Such vectors may comprise further genes such as marker genes which allow for the selection of said vector in a suitable host cell and under suitable conditions. Preferably, the polynucleotide of the invention is operatively linked to expression control sequences allowing expression in prokaryotic or eukaryotic cells. Expression of said polynucleotide comprises transcription of the polynucleotide into a translatable mRNA. Regulatory elements ensuring expression in eukaryotic cells, preferably mammalian cells, are well known to those skilled in the art. They usually comprise regulatory sequences ensuring initiation of transcription and optionally poly-A signals ensuring termination of transcription and stabilization of the transcript. Additional regulatory elements may include transcriptional as well as translational enhancers, and/or naturally-associated or heterologous promoter regions. Possible regulatory elements permitting expression in prokaryotic host cells comprise, e.g., the PL, lac, trp or tac promoter in E. coli, and examples for regulatory elements permitting expression in eukaryotic host cells are the AOX1 or GAL1 promoter in yeast or the CMV-, SV40- , RSV-promoter (Rous sarcoma virus), CMV-enhancer, SV40-enhancer or a globin intron in mammalian and other animal cells. Beside elements which are responsible for the initiation of transcription such regulatory elements may also comprise transcription termination signals, such as the SV40-poly-A site or the tk-poly-A site, downstream of the polynucleotide. Furthermore, depending on the expression system used leader sequences capable of directing the polypeptide to a cellular compartment or secreting it into the medium may be added to the coding sequence of the polynucleotide of the invention and are

well known in the art; see also, e.g., the appended examples. The leader sequence(s) is (are) assembled in appropriate phase with translation, initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein, or a portion thereof, into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product; see supra. In this context, suitable expression vectors are known in the art such as Okayama-Berg cDNA expression vector pcDV1 (Pharmacia), pCDM8, pRc/CMV, pcDNA1, pcDNA3 (In-vitrogene), or pSPORT1 (GIBCO BRL).

Preferably, the expression control sequences will be eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells, but control sequences for prokaryotic hosts may also be used. Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level expression of the nucleotide sequences, and, as desired, the collection and purification of the polypeptide of the invention may follow; see, e.g., the appended examples.

As described above, the polynucleotide of the invention can be used alone or as part of a vector to express the polypeptide of the invention in cells, for, e.g., gene therapy or diagnostics of diseases related to B-cell disorders. The polynucleotides or vectors containing the DNA sequence(s) encoding any one of the above described polypeptides is introduced into the cells which in turn produce the polypeptide of interest. Gene therapy, which is based on introducing therapeutic genes into cells by ex-vivo or in-vivo techniques is one of the most important applications of gene transfer. Suitable vectors and methods for in-vitro or in-vivo gene therapy are described in the literature and are known to the person skilled in the art; see, e.g., Giordano, Nature Medicine 2 (1996), 534-539; Schaper, Circ. Res. 79 (1996), 911-919; Anderson, Science 256 (1992), 808-813; Isner, Lancet 348 (1996), 370-374; Muhlhauser, Circ. Res. 77 (1995), 1077-1086; Wang, Nature Medicine 2 (1996), 714-716; WO94/29469; WO 97/00957 or Schaper, Current Opinion in Biotechnology 7 (1996), 635-640, and references cited therein. The polynucleotides and vectors of the invention may be designed for direct introduction or for introduction via

liposomes, or viral vectors (e.g. adenoviral, retroviral) into the cell. Preferably, said cell is a germ line cell, embryonic cell, or egg cell or derived therefrom, most preferably said cell is a stem cell.

In accordance with the above, the present invention relates to vectors, particularly plasmids, cosmids, viruses and bacteriophages used conventionally in genetic engineering that comprise a polynucleotide encoding a polypeptide of the invention. Preferably, said vector is an expression vector and/or a gene transfer or targeting vector. Expression vectors derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus, herpes viruses, or bovine papilloma virus, may be used for delivery of the polynucleotides or vector of the invention into targeted cell populations. Methods which are well known to those skilled in the art can be used to construct recombinant vectors; see, for example, the techniques described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989). Alternatively, the polynucleotides and vectors of the invention can be reconstituted into liposomes for delivery to target cells. The vectors containing the polynucleotides of the invention can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts; see Sambrook, supra. Once expressed, the polypeptides of the present invention can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like; see, Scopes, "Protein Purification", Springer-Verlag, N.Y. (1982). Substantially pure polypeptides of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity are most preferred, for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the polypeptides may then be used therapeutically (including extracorporeally) or in developing and performing assay procedures.

In a still further embodiment, the present invention relates to a cell containing the polynucleotide or vector described above. Preferably, said cell is a eukaryotic, most preferably a mammalian cell if therapeutic uses of the polypeptide are envisaged. Of

course, yeast and less preferred prokaryotic, e.g., bacterial cells may serve as well, in particular if the produced polypeptide is used as a diagnostic means.

The polynucleotide or vector of the invention which is present in the host cell may either be integrated into the genome of the host cell or it may be maintained extrachromosomally.

The term "prokaryotic" is meant to include all bacteria which can be transformed or transfected with a DNA or RNA molecules for the expression of a polypeptide of the invention. Prokaryotic hosts may include gram negative as well as gram positive bacteria such as, for example, E. coli, S. typhimurium, Serratia marcescens and Bacillus subtilis. The term "eukaryotic" is meant to include yeast, higher plant, insect and preferably mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue. A polynucleotide coding for a polypeptide of the invention can be used to transform or transfect the host using any of the techniques commonly known to those of ordinary skill in the art. Especially preferred is the use of a plasmid or a virus containing the coding sequence of the polypeptide of the invention and genetically fused thereto an N-terminal FLAG-tag and/or C-terminal His-tag. Preferably, the length of said FLAG-tag is about 4 to 8 amino acids, most preferably 8 amino acids. Methods for preparing fused, operably linked genes and expressing them in, e.g., mammalian cells and bacteria are well-known in the art (Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989). The genetic constructs and methods described therein can be utilized for expression of the polypeptide of the invention in eukaryotic or prokaryotic hosts. In general, expression vectors containing promoter sequences which facilitate the efficient transcription of the inserted polynucleotide are used in connection with the host. The expression vector typically contains an origin of replication, a promoter, and a terminator, as well as specific genes which are capable of providing phenotypic selection of the transformed cells. Furthermore, transgenic animals, preferably mammals, comprising cells of the invention may be used for the large scale production of the polypeptide of the invention.

In a further embodiment, the present invention thus relates to a process for the preparation of a polypeptide described above comprising cultivating a cell of the invention under conditions suitable for the expression of the polypeptide and isolating the polypeptide from the cell or the culture medium.

The transformed hosts can be grown in fermentors and cultured according to techniques known in the art to achieve optimal cell growth. The polypeptide of the invention can then be isolated from the growth medium, cellular lysates, or cellular membrane fractions. The isolation and purification of the, e.g., microbially expressed polypeptides of the invention may be by any conventional means such as, for example, preparative chromatographic separations and immunological separations such as those involving the use of monoclonal or polyclonal antibodies directed, e.g., against a tag of the polypeptide of the invention or as described in the appended examples.

Thus, the present invention allows the recombinant production of polypeptides comprising binding sites having affinity and specificity for an epitope of the CD19 and CD3 antigen, respectively, and optionally a further functional domain. As is evident from the foregoing, the invention provides a large family of polypeptides comprising such binding sites for any use in therapeutic and diagnostic approaches. It will be apparent to those skilled in the art that the polypeptides of the invention can be further coupled to other moieties as described above for, e.g., drug targeting and imaging applications. Such coupling may be conducted chemically after expression of the polypeptides to site of attachment or the coupling product may be engineered into the polypeptide of the invention at the DNA level. The DNAs are then expressed in a suitable host system, and the expressed proteins are collected and renatured, if necessary. As described above, the binding sites are preferably derived from the variable region of antibodies. In this respect, hybridoma technology enables production of cell lines secreting antibody to essentially any desired substance that produces an immune response. RNA encoding the light and heavy chains of the immunoglobulin can then be obtained from the cytoplasm of the hybridoma. The 5' end portion of the mRNA can be used to prepare cDNA to be used in the method of the present invention. The DNA encoding the polypeptides of the invention can subsequently be expressed in cells, preferably mammalian cells.

Depending on the host cell, renaturation techniques may be required to attain proper conformation. If necessary, point substitutions seeking to optimize binding may be made in the DNA using conventional cassette mutagenesis or other protein engineering methodology such as is disclosed herein. Preparation of the polypeptides of the invention may also be dependent on knowledge of the amino acid sequence (or corresponding DNA or RNA sequence) of bioactive proteins such as enzymes, toxins, growth factors, cell differentiation factors, receptors, anti-metabolites, hormones or various cytokines or lymphokines. Such sequences are reported in the literature and available through computerized data banks. For example, a polypeptide of the invention can be constructed that, e.g., consists of the single-chain Fv fragment and the extracellular part of the human costimulatory protein CD80 (B7-1) connected by a (Gly4Ser1)1 linker. The CD80 costimulatory protein belongs to the Ig superfamily. It is a heavily glycosylated protein of 262 amino acids. A more detailed description was published by Freeman G.J et.al. J.Immunol.143,(1989) 2714 - 2722. Stable expression can be performed in, e.g., DHFR deficient CHO-cells as described by Kaufmann R.J. (1990) Methods Enzymol. 185, 537-566. The protein can then be purified via its His-tag attached to the C-terminus by using a Ni-NTA-column (Mack, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 7021-7025).

Moreover, the present invention relates to pharmaceutical compositions comprising the aforementioned polypeptide, the polynucleotide or the vector of the invention.
The pharmaceutical composition of the present invention may further comprise a pharmaceutically acceptable carrier. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by well known conventional methods. These pharmaceutical compositions can be administered to the subject at a suitable dose. Administration of the suitable compositions may be effected by different ways, e.g., by intravenous, intraperitoneal, subcutaneous, intramuscular, topical or intradermal administration. The dosage regimen will be determined by the attending physician and clinical factors. As is well

known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Generally, the regimen as a regular administration of the pharmaceutical composition should be in the range of 1 µg to 10 mg units per day. If the regimen is a continuous infusion, it should also be in the range of 1 µg to 10 mg units per kilogram of body weight per minute, respectively. Progress can be monitored by periodic assessment. Dosages will vary but a preferred dosage for intravenous administration of DNA is from approximately 10^6 to 10^{12} copies of the DNA molecule. The compositions of the invention may be administered locally or systemically. Administration will generally be parenterally, e.g., intravenously; DNA may also be administered directly to the target site, e.g., by biolistic delivery to an internal or external target site or by catheter to a site in an artery. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

Furthermore, the pharmaceutical composition of the invention may comprise further agents such as T-cell co-stimulatory molecules or cytokines known in the art.

It is envisaged by the present invention that the various polynucleotides and vectors of the invention are administered either alone or in any combination using standard vectors and/or gene delivery systems, and optionally together with a pharmaceutically acceptable carrier or excipient. Subsequent to administration, said polynucleotides or vectors may be stably integrated into the genome of the subject.

On the other hand, viral vectors may be used which are specific for certain cells or tissues and persist in said cells. Suitable pharmaceutical carriers and excipients are well known in the art. The pharmaceutical compositions prepared according to the invention can be used for the prevention or treatment or delaying of different kinds of diseases, which are related to B-cell related immunodeficiencies and malignancies.

Furthermore, it is possible to use a pharmaceutical composition of the invention which comprises polynucleotide or vector of the invention in gene therapy. Suitable gene delivery systems may include liposomes, receptor-mediated delivery systems, naked DNA, and viral vectors such as herpes viruses, retroviruses, adenoviruses, and adeno-associated viruses, among others. Delivery of nucleic acids to a specific site in the body for gene therapy may also be accomplished using a biolistic delivery system, such as that described by Williams (Proc. Natl. Acad. Sci. USA 88 (1991), 2726-2729).

It is to be understood that the introduced polynucleotides and vectors express the gene product after introduction into said cell and preferably remain in this status during the lifetime of said cell. For example, cell lines which stably express the polynucleotide under the control of appropriate regulatory sequences may be engineered according to methods well known to those skilled in the art. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the polynucleotide of the invention and a selectable marker, either on the same or separate plasmids. Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows for the selection of cells having stably integrated the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. Such engineered cell lines are also particularly useful in screening methods for the detection of compounds involved in, e.g., B-cell/T-cell interaction.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, Cell 11(1977), 223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska, Proc. Natl. Acad. Sci. USA 48 (1962), 2026), and adenine phosphoribosyltransferase (Lowy, Cell 22 (1980), 817) in tk^-, $hgprt^-$ or

aprt⁻ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, Proc. Natl. Acad. Sci. USA 77 (1980), 3567; O'Hare, Proc. Natl. Acad. Sci. USA 78 (1981), 1527), gpt, which confers resistance to mycophenolic acid (Mulligan, Proc. Natl. Acad. Sci. USA 78 (1981), 2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, J. Mol. Biol. 150 (1981), 1); hygro, which confers resistance to hygromycin (Santerre, Gene 30 (1984), 147); or puromycin (pat, puromycin N-acetyl transferase). Additional selectable genes have been described, for example, trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman, Proc. Natl. Acad. Sci. USA 85 (1988), 8047); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.).

In another embodiment the present invention relates to a diagnostic composition comprising any one of the above described the polypeptides, polynucleotides or vectors of the invention and optionally suitable means for detection.

The polypeptides of the invention are also suited for use in immunoassays in which they can be utilized in liquid phase or bound to a solid phase carrier. Examples of immunoassays which can utilize the polypeptide of the invention are competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such immunoassays are the radioimmunoassay (RIA), the sandwich (immunometric assay) and the Western blot assay.

The polypeptides of the invention can be bound to many different carriers and used to isolate cells specifically bound to said polypeptides. Examples of well-known carriers include glass, polystyrene, polyvinyl chloride, polypropylene, polyethylene, polycarbonate, dextran, nylon, amyloses, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble or insoluble for the purposes of the invention.

There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, colloidal metals, fluorescent compounds, chemiluminescent compounds, and bioluminescent compounds; see also the embodiments discussed hereinabove.

The present invention also relates to the use of the polypeptide, polynucleotide and vector of the invention described hereinabove for the preparation of a pharmaceutical composition for the treatment of B-cell malignancies, B-cell mediated autoimmune diseases or the depletion of B-cells.

Recent clinical studies with retargeted cytotoxic activity of human T cells by bispecific antibodies have shown promising results in the treatment of refractory Hodgkin's desease (33), breast and ovarian cancer (34-37) and malignant glioma (38). Given the facts

- that bsc antibodies due to their low molecular mass facilitate penetration into tumors (as has been shown for Fab or Fv fragments) (39); and
- that bsc antibodies are suspected to decrease the dose dependent and dose limiting toxicity caused by the systemic cytokine release mediated by the Fc parts of conventional bispecific antibodies (40); and
- that even an intact monoclonal antibody (directed against CD20) led to tumor regression in advanced stages of NHL (41, 42),

it is expected that the polypeptides of the invention are interesting molecules that contribute to further therapeutic improvements.

Thus, in a preferred embodiment the pharmaceutical composition of the invention is used for the treatment of non-Hodgkin lymphoma.

The dosage ranges for the administration of the polypeptides, polynucleotides and vectors of the invention are those large enough to produce the desired effect in which the symptoms of the B-cell mediated diseases are ameliorated. The dosage should not be so large as to cause essential adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex, and extent of the disease in the patient and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any counterindications.

Furthermore, the invention relates to a method for identifying T-cell activating or co-stimulating compounds or for identifying inhibitors of T-cell activation and stimulation comprising

(a) culturing B- and T-cells in the presence of the polypeptide of the invention and, optionally, in the presence of a component capable of providing a detectable signal in response to T-cell activation with a compound to be screened under conditions permitting interaction of the compound with the cells; and

(b) detecting the presence or absence of a signal generated from the intention of the compound with the cells.

The term "compound" in the method of the invention includes a single substance or a plurality of substances which may or may not be identical.

Said compound(s) may be comprised in, for example, samples, e.g., cell extracts from, e.g., plants, animals or microorganisms. Furthermore, said compounds may be known in the art but hitherto not known to be capable of inhibiting T-cell activation or not known to be useful as a T-cell co-stimulatory factor, respectively. The plurality of compounds may be, e.g., added to the culture medium or injected into the cell.

If a sample containing (a) compound(s) is identified in the method of the invention, then it is either possible to isolate the compound from the original sample identified as containing the compound, in question or one can further subdivide the original sample, for example, if it consists of a plurality of different compounds, so as to reduce the number of different substances per sample and repeat the method with the subdivisions of the original sample. It can then be determined whether said sample or compound displays the desired properties by methods known in the art such as described herein and in the appended examples. Depending on the complexity of the samples, the steps described above can be performed several times, preferably until the sample identified according to the method of the invention only comprises a limited number of or only one substance(s). Preferably said sample comprises substances of similar chemical and/or physical properties, and most preferably said substances are identical. The methods of the present invention can be easily performed and designed by the person skilled in the art, for example in accordance with other cell based assays described in the prior art or by using and

modifying the methods as described in the appended examples. Furthermore, the person skilled in the art will readily recognize which further compounds and/or cells may be used in order to perform the methods of the invention, for example, interleukins, or enzymes, if necessary, that convert a certain compound into the precursor which in turn stimulates or suppresses T-cell activation. Such adaptation of the method of the invention is well within the skill of the person skilled in the art and can be performed without undue experimentation.

Compounds which can be used in accordance with the method of the present invention include peptides, proteins, nucleic acids, antibodies, small organic compounds, ligands, peptidomimetics, PNAs and the like. Said compounds can also be functional derivatives or analogues of known T-cell activators or inhibitors. Methods for the preparation of chemical derivatives and analogues are well known to those skilled in the art and are described in, for example, Beilstein, Handbook of Organic Chemistry, Springer edition New York Inc., 175 Fifth Avenue, New York, N.Y. 10010 U.S.A. and Organic Synthesis, Wiley, New York, USA. Furthermore, said derivatives and analogues can be tested for their effects according to methods known in the art or as described, for example, in the appended examples. Furthermore, peptidomimetics and/or computer aided design of appropriate activators or inhibitors of T-cell activation can be used, for example, according to the methods described below. Appropriate computer programs can be used for the identification of interactive sites of a putative inhibitor and the antigen of the invention by computer assistant searches for complementary structural motifs (Fassina, Immunomethods 5 (1994), 114-120). Further appropriate computer systems for the computer aided design of protein and peptides are described in the prior art, for example, in Berry, Biochem. Soc. Trans. 22 (1994), 1033-1036; Wodak, Ann. N. Y. Acad. Sci. 501 (1987), 1-13; Pabo, Biochemistry 25 (1986), 5987-5991. The results obtained from the above-described computer analysis can be used in combination with the method of the invention for, e.g., optimizing known T-cell activators or inhibitors. Appropriate peptidomimetics can also be identified by the synthesis of peptidomimetic combinatorial libraries through successive chemical modification and testing the resulting compounds, e.g., according to the methods described herein and in the appended examples. Methods for the generation and use of

peptidomimetic combinatorial libraries are described in the prior art, for example in Ostresh, Methods in Enzymology 267 (1996), 220-234 and Dorner, Bioorg. Med. Chem. 4 (1996), 709-715. Furthermore, the three-dimensional and/or crystallographic structure of inhibitors or activators of B-cell/T-cell interaction can be used for the design of peptidomimetic inhibitors or activators of T-cell activation to be tested in the method of the invention (Rose, Biochemistry 35 (1996), 12933-12944; Rutenber, Bioorg. Med. Chem. 4 (1996), 1545-1558).

In summary, the present invention provides methods for identifying compounds which are capable of modulating B-cell/T-cell mediated immune responses. Compounds found to activate B-cell/T-cell mediated responses may be used in the treatment of cancer and related diseases. In addition, it may also be possible to specifically inhibit viral diseases, thereby preventing viral infection or viral spread. Compounds identified as suppressors of T-cell activation or stimulation may be used in organ transplantation in order to avoid graft rejection; see also supra.
The compounds identified or obtained according to the method of the present invention are thus expected to be very useful in diagnostic and in particular for therapeutic applications. Hence, in a further embodiment the invention relates to a method for the production of a pharmaceutical composition comprising formulating the compound identified in step (b) of the above described methods of the invention in a pharmaceutically acceptable form.
The therapeutically useful compounds identified according to the method of the invention may be administered to a patient by any appropriate method for the particular compound, e.g., orally, intravenously, parenterally, transdermally, transmucosally, or by surgery or implantation (e.g., with the compound being in the form of a solid or semi-solid biologically compatible and resorbable matrix) at or near the site where the effect of the compound is desired. Therapeutic doses are determined to be appropriate by one skilled in the art, see supra.

These and other embodiments are disclosed and encompassed by the description and Examples of the present invention. Further literature concerning any one of the antibodies, methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries and databases, using for

example electronic devices. For example the public database "Medline" may be utilized which is available on the Internet, for example under http://www.ncbi.nlm.nih.gov/PubMed/medline.html. Further databases and addresses, such as http://www.ncbi.nlm.nih.gov/, http://www.infobiogen.fr/, http://www.fmi.ch/biology/research_tools.html, http://www.tigr.org/, are known to the person skilled in the art and can also be obtained using, e.g., http://www.lycos.com. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

The figures show:

Figure 1:

SDS-Page: Coomassie stain of the purified bsCD19xCD3 fragment with different amounts of protein. Molecular mass (kDa) of the marker is indicated on the left.

Figure 2:

FACS-analysis with the bscCD19xCD3 (200µg/ml) on different CD19-positive B cell lines (BJAB, SKW6.4, Blin-1, Daudi, Raji), on the CD19-negative B cell line BL60 and on CD3-positve Jurkat cells and primary human PBMCs. Broken lines indicate negative controls.

Figure 3:

Cytotoxicity of bscDC19xCD3 in a ^{51}Cr release assay with unstimulated human PBMCs and different B cell lines. Effector:Target cell ratio 10:1; incubation time 4 h. Standard deviation in all triplicates was below 7%.

Figure 4:

Chromium release cytotoxicity assay with unstimulated primary human PBLs against the plasmacytoma cell lines L363 and NCI and the lymphoma cell line Daudi E:T ratio 20:1; incubation time 8h

Figure 5:

Inhibition of the cytotoxicity of bscCD19xCD3 by the parental anti-CD19 antibody HD37 in a chromium release assay; incubation time 8h; E:T ratio 20:1; concentration of bscCD19xCD3 1ng/ml

Figure 6:

Cytotoxicity assay with unstimulated PBMC´s against Daudi cells after addition of increasing amounts of EGTA, E:T ration 10:1, incubation time 4 h

Figure 7:

Cytotoxicity of bscCD19xCD3 in a ^{51}Cr release assay with unstimulated human PBMCs and Blin-1 as target cells at different E:T ratios; incubation time 4 h; concentration of the conventional bispecific antibody 3µg/ml; concentration of bsc 17-1AxCD3 100 ng/ml; E:T ratios as indicated

Figure 8:

DNA- and protein-sequence of the bscCD19xCD3 antibody (FLAG-tag containing variant). Numbers indicate the nucleotide (nt) postions, the corresponding amino acid sequence is depicted below the nucleotide sequence. The encoding DNA sequence for the bispecific antibody starts at position 1 and ends at position 1593. The first six nt (position -10 to -5) and the last six nt (position 1596 to 1601) contain the restriction enzyme cleavage sites for EcoRI and SalI, respectively. Nucleotides 1 to 57 specify the leader sequence; nucleotides 82 to 414 and 460 to 831 encode V_LCD19 and V_HCD19, respectively; nucleotides 847 to 1203 and 1258 to 1575 encode V_HCD3 and V_LCD3, respectively; and nucleotides 1576 to 1593 encode a His-tag.

The following Examples illustrate the invention:

Example 1: Cloning of Variable (V) Immunoglobulin Domains

The V light-chain (VL) and V heavy-chain (VH) domains from the HD37 hybridoma (22) were cloned according to standard PCR methods (23). cDNA synthesis was carried out with oligo dT primers and Taq polymerase.

List of Primers

5´ L1:

GAAGCACGCGTAGATATCG/TTG(AC)T(GC)ACCCAA(TA)CTCCA

3´ K:

GAAGATGGATCCAGCGGCCGCAGCATCAGC

5´ H1:

CAGCCGGCCATGGCGCAGGT(CG)CAGCTGCAG(CG)AG

3´ G:

ACCAGGGGCCAGTGGATAGACAAGCTTGGGTGTCGTTTT

5´VLB5RRV:

AGGTGTACACTCCGATATCCAGCTGACCCAGTCTCCA

3´VLGS15:

GGAGCCGCCGCCGCCAGAACCACCACCACCTTTGATCTCGAGCTTGGTCCC

5´VHGS15:

GGCGGCGGCGGCTCCGGTGGTGGTGGTTCTCAGGT(GC)(AC)A(AG)CTGCAG(GC)AGTC(AT)GG

3´VHBspE1:

AATCCGGAGGAGACGGTGACCGTGGTCCCTTGGCCCCAG

For the amplification of the V domains via PCR we used the primers 5´ L1 and 3´ K, flanking the VL, domain, and 5´H1 and 3´G for the heavy chain based on primers described by Dübel *et al.*. (24).

The cDNA of the anti-CD3 scFv fragment was kindly provided by A. Traunecker (25).

Example 2: Construction of Bispecific Single-Chain Fragments and Eukaryotic Expression

To obtain an anti-CD19 scFv-fragment, the corresponding VL- and VH-regions cloned into separate plasmid vectors served as templates for a VL- and VH-specific PCR using the oligonucleotide primer pairs 5'VLB5RRV/3'VLGS15 and 5'VHGS15/3'VHBspEI, respectively. Thereby, overlapping complementary sequences were introduced into the PCR-products, that combine to form the coding sequence of 15-amino acid $(Gly_4Ser_1)_3$-linker during the subsequent fusion-PCR. This amplification step was performed with the primer pair 5'VLB5RRV/3'VHBspEI and the resulting fusion product (or rather anti-CD19 scFv-fragment) was cleaved with the restriction enzymes EcoRV and BspEI and thus cloned into the bluescript KS-vector (Stratagene) containing either the (EcoRI/SalI-cloned) coding sequence of the anti-17-1A/anti-CD3 bispecific single-chain antibody with an N-terminal FLAG-tag [1] or that of the modified version without FLAG-eptiope (40), thereby replacing the anti-17-1A- by the anti-CD19-specificity and preserving the 5- amino acid $(Gly_4Ser_1)_1$-linker connecting the C-terminal anti-CD3 scFv-fragment, respectively. Subsequently, the DNA fragments encoding both versions of the anti-CD19/anti-CD3 bispecific single-chain antibody with the domain arrangement VL_{CD19}-VH_{CD19}-VH_{CD3}-VL_{CD3} were subcloned EcoRI/SalI into the described expression vector pEF-DHFR [1], respectively. The resulting plasmid DNAs were transfected into DHFR-deficient CHO-cells by electorporation: selection, gene amplificationn and protein production were performed as described [1].

Purification of bscCD19xCD3 from the supernatant of transfected CHO cells yielded 4 mg/liter culture supernatant. The bsc-Ab was purified via ist C-terminal histidine tail by affinity chromatography on a Ni-NTA-column as described [1]. The bsc-Ab was eluted from the Ni-NTA column as a distinct peak at a concentration of 200 mM imidazole. SDS-Page was carried out according to Laemmli (26) with a 12% gel followed by staining with Coomassie brilliant blue R250 for analysing the purification of the bsc-Ab. The results of SDS/PAGE analysis (Fig. 1) show the expected size of the bsc-Ab (60 kDa).

Example 3: Binding properties of the bsc-AbCD19xCD3

Binding specificities of the bsc-Ab to CD3 and CD19 were shown by flow cytometric analysis on CD3-positive Jurkat cells, human PBMCs and a number of different CD19-positive B cell lymphoma cell lines including Blin I, SKW6.4, Daudi, BJAB and Raji. The CD19-positive B cell lines Daudi, Raji, BJAB (Burkitt's lymphoma), SKW6.4 (human EBV transformed B cell) and Blin-1 (pre B cell line) were used in flow cytometric analysis and chromium release assays. Jurkat is a CD3-positive T cell line; BL60 and the plasmacytoma cell lines NCI and L363 are negative for both surface molecules, CD3 and CD19. Cell lines were cultured in complete RPMI 1640 (Biochrom) with 10 % FCS (GIBCO).

1 x 10^6 cells were washed with PBS, resuspended in 200 µl PBS with 10 % Venimmun (Centeon, Marburg, Germany) and 0,1 % NaN_3 and incubated for 30 min at 4°C. After a centrifugation step (100 x g, 5 min) cells were incubated in 50 µl bscCD19xCD3 (200 µg/ml in PBS with 10 % Venimmun and 0,1 % NaN_3) for 30 min at 4°C. The cells were washed twice with PBS. For the detection of the bsc-Ab a FITC-conjugated antibody against the His-tag (Dianova) was used. The irrelevant bsc-Ab 17-1AxCD3, produced by the same expression system as bscCD19xCD3, or the His-tag antibody alone served as negative controls. Flow Cytometry was performed with a Becton Dickinson FACScan. No binding was detectable on BL60 cells which do express neither CD19 nor CD3 (Fig. 2).

Example 4: Cytotoxic activity of the bsc-AbCD19xCD3 against CD19-positive lymphoma cells

The bscCD19xCD3 antibody proved to be highly cytotoxic for several lymphoma cell lines in a ^{51}Cr release assay (Figure 3). Human peripheral blood mononuclear cells (PBMCs) as effector cells were isolated from fresh buffy coats of random donors using Lymphoprep™ (Nycomed) gradient centrifugation with subsequent 100 x g centrifugation steps to remove thrombocytes. CD19-positive B cells were depleted using Dynabeads® M-450 CD19 (Dynal). The depleted cell populations were analysed by flow cytometry (Becton Dickinson), which showed a 99% depletion of

CD19-positive cells. The PBMCs were incubated over night at 37°C, 5% CO_2. CD19-positive B cell lines (Raji, Blin I, Daudi, BJAB, SKW6.4) were used as target cells. Cytotoxicity was measured in a standard chromium release assay in round-bottom 96-well-plates (Nunc) using RPMI 1640 complete medium (Biochrom) with 10% FCS (GIBCO).

Unstimulated PBMCs were added in a volume of 80 µl medium to each well containing 20 µl of bsc-Ab in different concentrations. Then 100 µl of ^{51}Cr-labeled target cells (1 x 10^4) were added, plates were centrifuged for 3 min at 100 x g and incubated for 4 h at 37°C, 5 % CO_2. After an additional centrifugation step 50 µl supernatant was removed and assayed for released ^{51}Cr in a gamma counter (TopCount, Canberra Packard).

Spontaneous release was measured by incubating the target cells without effector cells or antibodies, and maximal release was determined by incubating the target cells with 10 % TritonX-100. Incubation of target cells with bsc-Ab without effector cells did not result in measurable lysis. The percentage specific lysis was calculated asspecific release (%)= [(cpm, experimental release) - (cpm, spontaneous release)] / [(cpm, maximal release) - (cpm, spontaneous release)] x 100. All tests were carried out in triplicates. SD within the triplicates was in all experiments below 6%. To approximate the *in vivo* conditions we used unstimulated PBMCs from healthy donors as effector cells. Rapid induction of cytotoxicity within 4 hours could be observed without any T cell prestimulation protocol. As a control a bsc-antibody with different tumor specificity (bsc17-1AxCD3) but generated by the same system as the bscCD19xCD3 antibody showed lysis activity not significantly above medium background. In addition, no cytotoxic activity could be observed using the plasmacytoma cell lines NCI and L363 which do not express CD19 as target cells (Figure 4). In competition assays using increasing amounts of the CD19-specific parental monoclonal antibody HD37 cytotoxic activity of the bscCD19xCD3 could be nearly completely blocked (Figure 5). These controls show that bscCD19xCD3-mediated cytotoxic effects are antigen-specific. To get more information about the molecular mechanisms how the bscCD19x CD3 antibody kills CD19-positive target cells we tried to block bscCD19xCD3-mediated cytotoxicity by EGTA. As shown in Figure 6 cytotoxic activity of bscCD19xCD3 could be completely blocked by EGTA

indicating that specific lysis is a T cell-mediated effect (probably via the perforin-pathway) rather than a direct (e.g. apoptosis-inducing) effect of the antibody itself. Using unstimulated T cells even at antibody concentrations below 1 ng/ml a significant cytotoxic effect against Blin-1 cells could be observed (Figure 7). Even at relatively low E:T ratios (5:1; 2.5:1) and at very low antibody concentrations of 10-100 pg/ml the bscCD19xCD3 antibody could rapidly induce specific cytotoxic activity of unstimulated T cells (Figure 7). In contrast, a conventional bispecific CD19xCD3 antibody generated by hybrid-hybridoma technique (5-7, 27) did not show significant cytotoxic activity under these conditions even at concentrations up to 3000ng/ml (Figure 7). This conventional bispecific antibody required additional T cell prestimulation and high antibody concentrations of about 100 ng/ml to induce specific T cell cytotoxicity (not shown) which is consistent with the literature (5-7, 27).

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CLAIMS

1. A single-chain multi-functional polypeptide comprising
   (a) a first domain comprising a binding-site of an immunoglobulin chain or an antibody specifically recognizing the CD19 antigen; and
   (b) a second domain comprising a binding site of an immunoglobulin chain or an antibody specifically recognizing the CD3 antigen.

2. The polypeptide of claim 1, wherein said two domains are connected by a polypeptide linker.

3. The polypeptide of claim 1 or 2, wherein said first and/or second domain mimic a V_H and V_L region from a natural antibody.

4. The polypeptide of any one of claims 1 to 3, wherein said antibody is monoclonal antibody, synthetic antibody, or humanized antibody.

5. The polypeptide of any one of claims 1 to 4, wherein at least one of said domains is a single-chain fragment of the variable region of the antibody.

6. The polypeptide of any one of claims 1 to 5, wherein said domains are arranged in the order V_L CD19 - V_HCD19 – V_HCD3 – V_LCD3.

7. The polypeptide of any one of claims 2 to 6, wherein said polypeptide linker comprises a plurality of glycine, alanine and/or serine residues.

8. The polypeptide of any one of claims 2 to 7, wherein said polypeptide linker comprises a plurality of consecutive copies of an amino acid sequence.

9. The polypeptide of any one of claims 2 to 8, wherein said polypeptide linker comprises 1 to 5 amino acid residues.

10. The polypeptide of any one of claims 2 to 9, wherein said polypeptide linker comprises the amino acid sequence Gly Gly Gly Gly Ser.

11. The polypeptide of any one of claims 1 to 10, wherein said first domain comprises at least one CDR of the V_H and V_L region comprising the amino acid sequence encoded by the DNA sequence depicted in Figure 8 from nucleotides 82 to 414 (V_L) and nucleotides 460 to 831 (V_H) and/or wherein said second domain comprises at least one CDR of the V_H and V_L region comprising the amino acid sequence encoded by the DNA sequence depicted in Figure 8 from nucleotides 847 to 1203 (V_H) and nucleotides 1258 to 1575 (V_L).

12. The polypeptide of any one of claims 1 to 11, wherein
    (a) said binding site of the first domain has an affinity of at least about 10^{-7} M; and/or
    (b) said binding site of the second domain has an affinity of less than about 10^{-7} M.

13. The polypeptide of any one of claims 1 to 12 that is a bispecific single-chain antibody.

15. The polypeptide of any one of claims 1 to 13, comprising at least one further domain.

15. The polypeptide of claim 14, wherein said further domain is linked by covalent or non-covalent bonds.

16. The polypeptide of claim 14 or 15, wherein said at least one further domain comprises an effector molecule having a conformation suitable for biological activity, capable of sequestering an ion or selective binding to a solid support or to a preselected determinant.

17. A polynucleotide which upon expression encodes a polypeptide of any one of claims 1 to 16.

18. A vector comprising the polynucleotide of claim 17.

19. A cell transfected with the polynucleotide of claim 17 or the vector of claim 18.

20. A method for the preparation of the polypeptide of any one of claims 1 to 16 which process comprises cultivating a cell of claim 19 and isolating said polypeptide from the culture.

21. A pharmaceutical composition comprising the polypeptide of any one of claims 1 to 16, the polynucleotide of claim 17 or the vector of claim 18 and optionally a pharmaceutically acceptable carrier.

22. A diagnostic composition comprising the polypeptide of any one of claims 1 to 16, the polynucleotide of claim 17 or the vector of claim 18 and optionally suitable means for detections.

23. Use of the polypeptide of any one of claims 1 to 16, the polynucleotide of claim 17 or the vector of claim 18 for the preparation of a pharmaceutical composition for the treatment of B-cell malignancies, B-cell mediated autoimmune diseases or the depletion of B-cells.

24. The use of claim 23, wherein said B-cell malignancy is non-Hodgkin lymphoma.

25. Use of the polynucleotide of claim 17 or the vector of claim 18 for the preparation of compositions for gene therapy.

26. A method for identifying activators or inhibitors of T-cell activation or stimulation comprising

(a) culturing T-cells and B-cells in the presence of a polypeptide of any one of claims 1 to 16 and optionally in the presence of a component capable of providing a detectable signal in response to T-cell activation with a compound to be screened under conditions to permit activation of the T-cell, and

(b) detecting the presence or absence of the signal generated from the interaction of the compound with the cells.

27. A method for the production of a pharmaceutical composition comprising the steps of the method of claim 26 and formulating the compound identified in step (b) in a pharmaceutically acceptable form.

ABSTRACT

Described are novel single-chain multifunctional polypeptides comprising at least two binding sites specific for the CD19 and CD3 antigen, respectively. Further provided are polypeptides, wherein the above-described polypeptide comprises at least one further domain, preferably of pre-determined function. Furthermore, polynucleotides encoding said polypeptides as well as to vectors comprising said polynucleotides and host cells transformed therewith and their use in the production of said polypeptides are described. In addition, pharmaceutical and diagnostic compositions are provided comprising any of the afore-described polypeptides, polynucleotides or vectors. Described is also the use of the afore-mentioned polypeptides, polynucleotides and vectors for the preparation of pharmaceutical compositions for immunotherapy, preferably against B-cell malignancies such as non-Hodgkin lymphoma.

Figure 1:

SDS-Page: Coomassie stain of the purified bscCD19xCD3 fragment

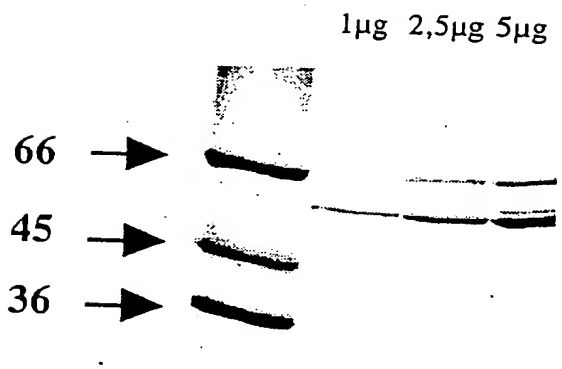
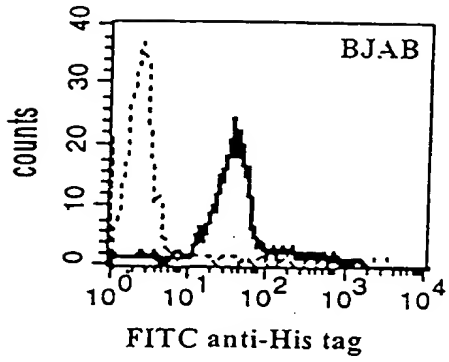


Figure 2: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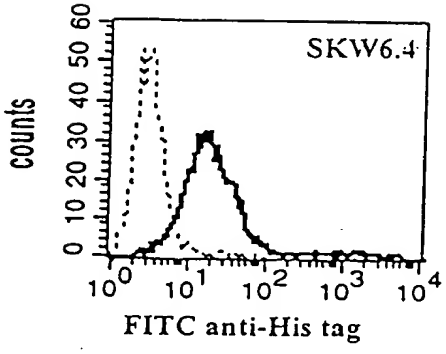
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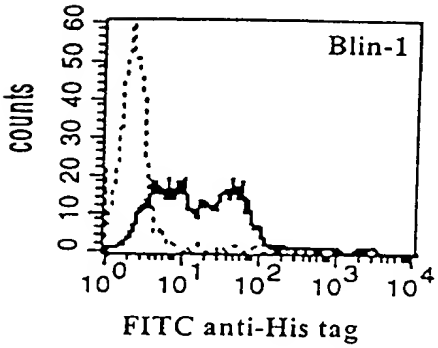
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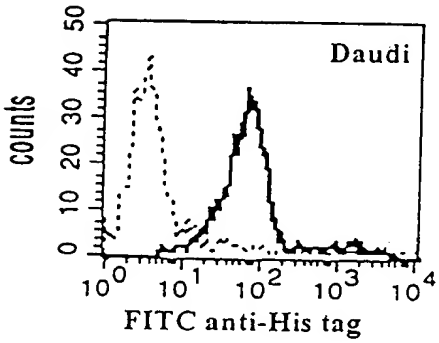
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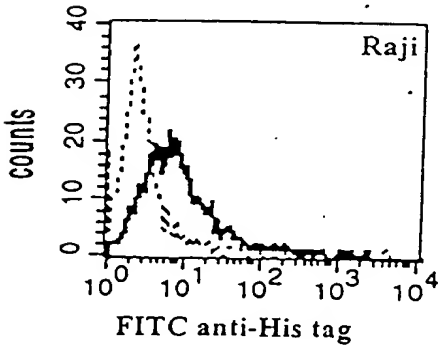
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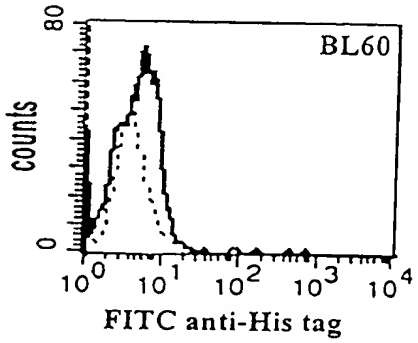
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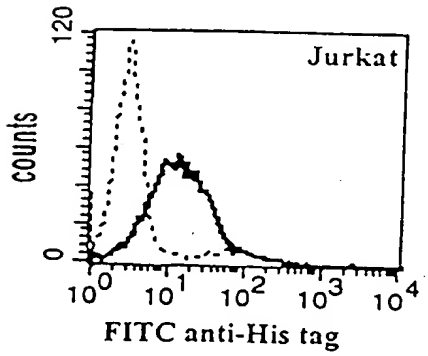
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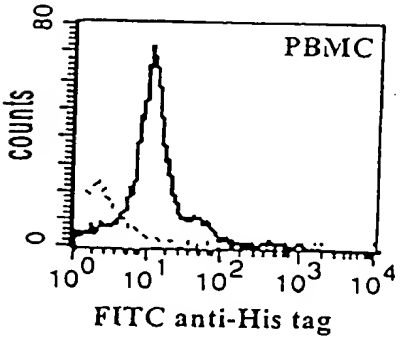
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Figure 3:

Cytotoxicity of bscCD19xCD3 in a ^{51}Cr release assay with unstimulated PBMC against different B cell lines

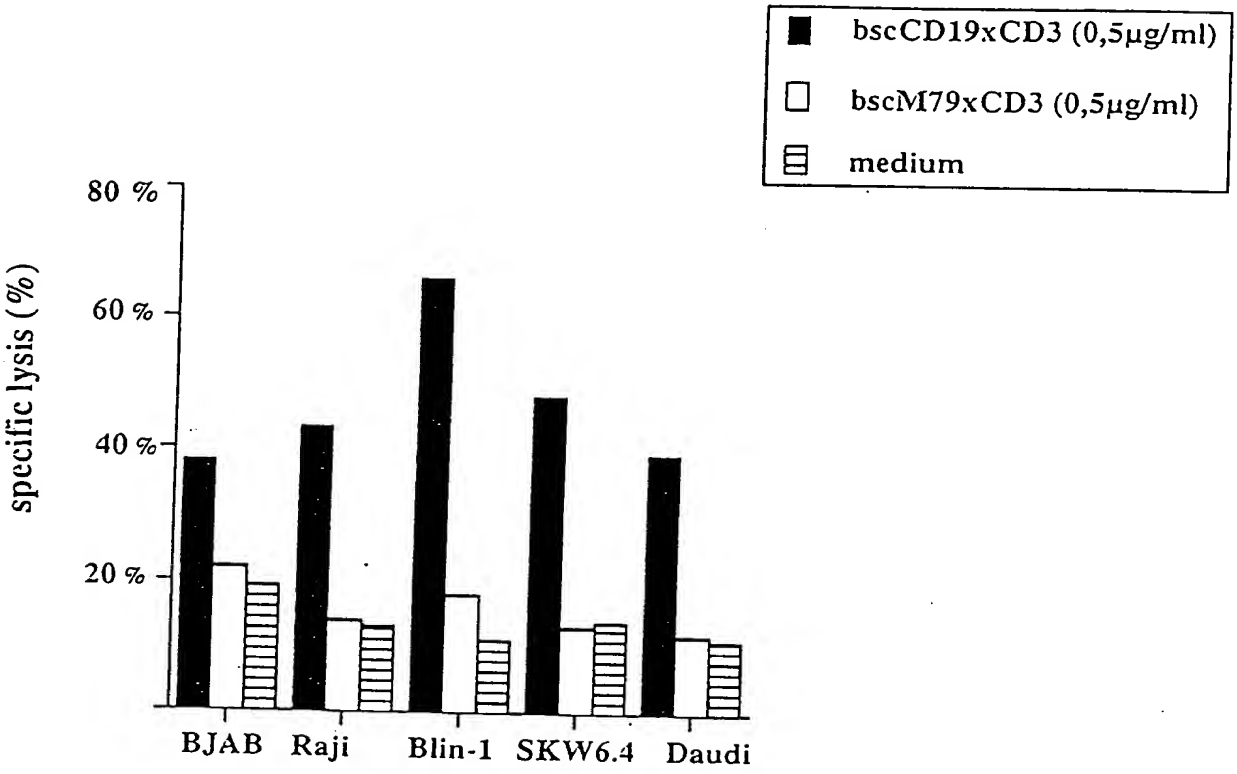


Figure 4:

Cytotoxicity assay with PBMC against CD19$^+$ Daudi and CD19$^-$ plasmacytoma cells (L363 and NCI)

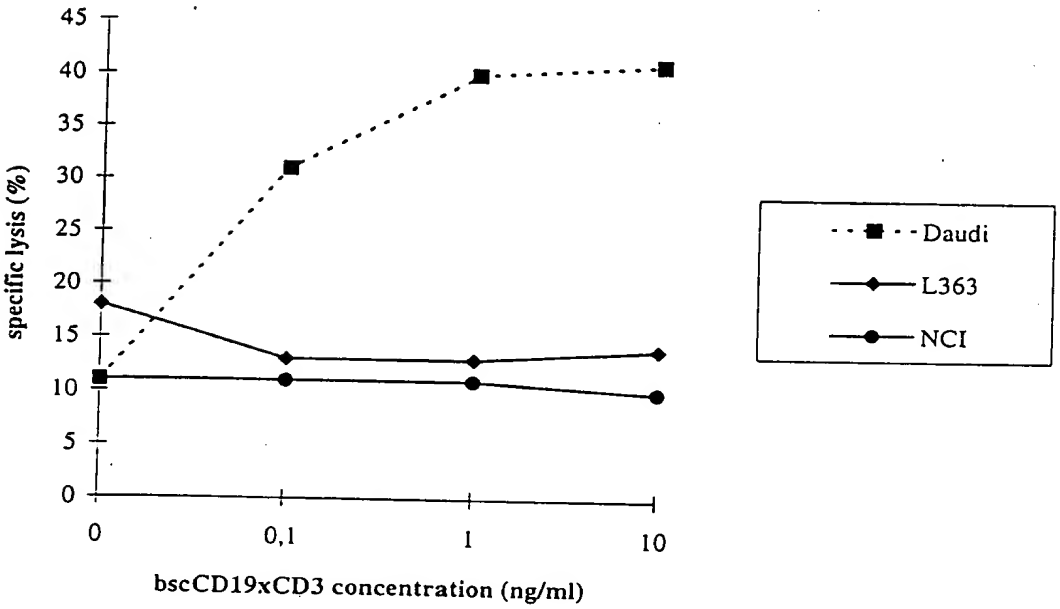


Figure 5:

Inhibition of the cytotoxicity of bscCD19xCD3 by the parental anti-CD19 antibody HD37

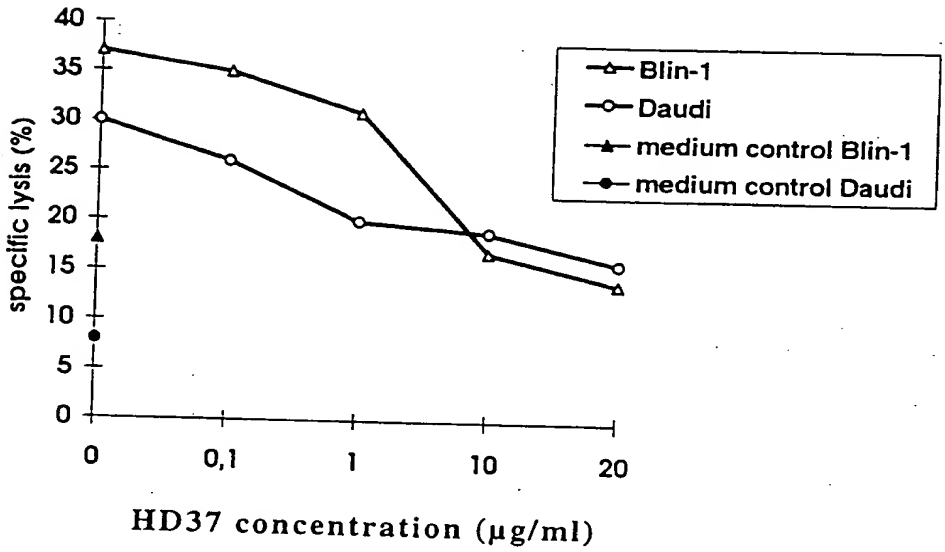


Figure 6:

Cytotoxicity assay with unstimulated PBMC´s against Daudi cells after addition of increasing amounts of EGTA

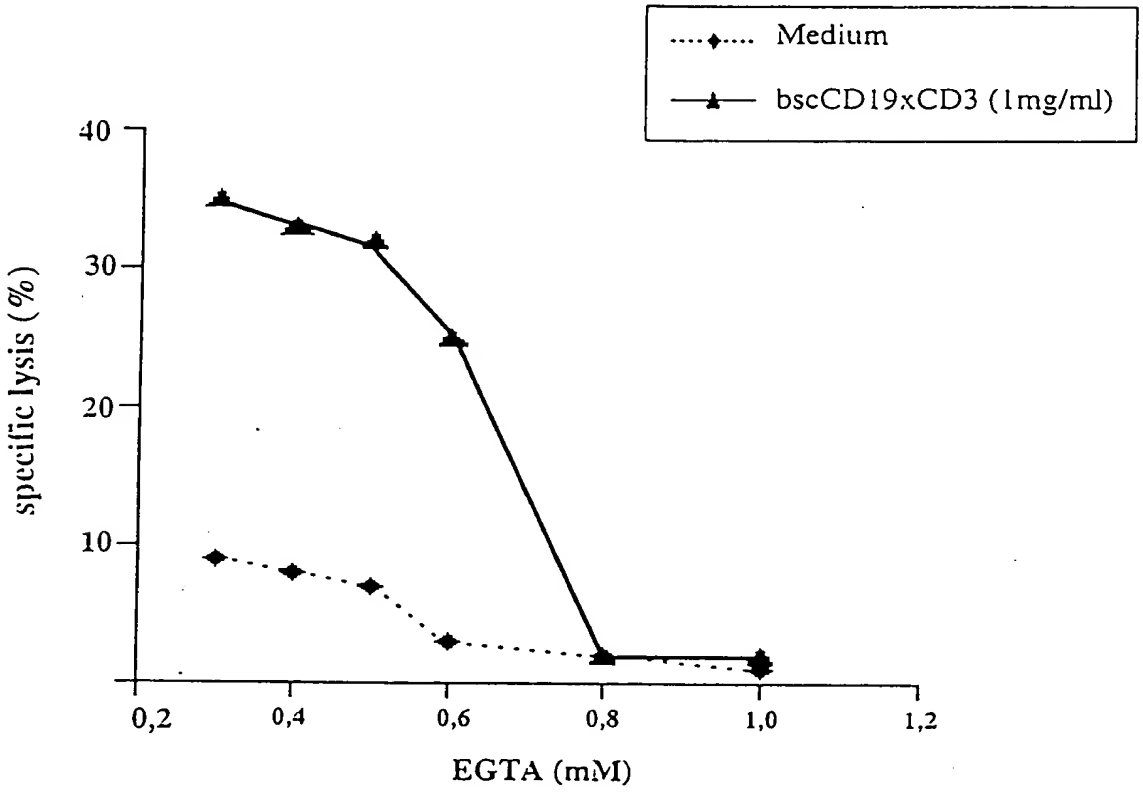


Figure 7:

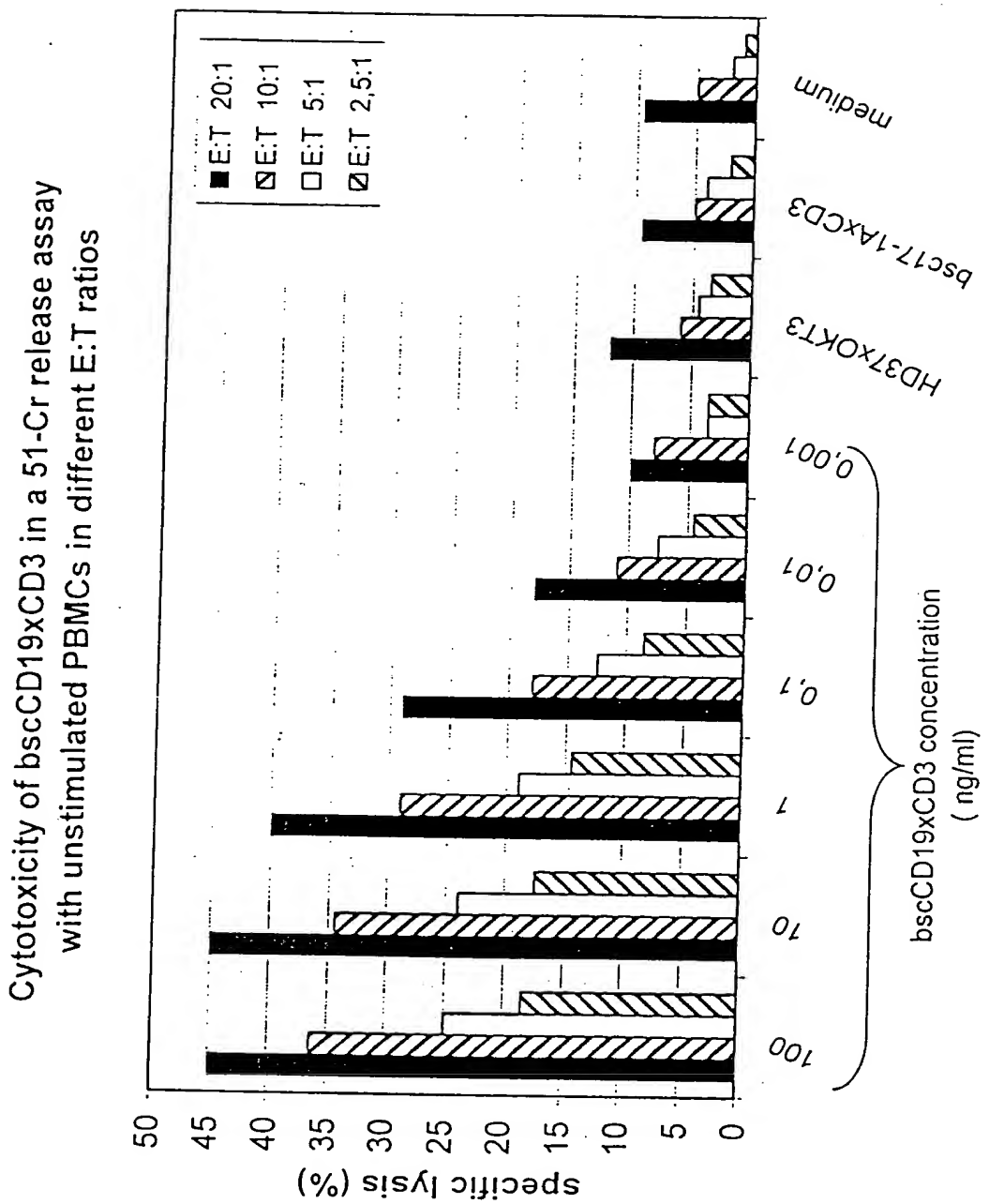
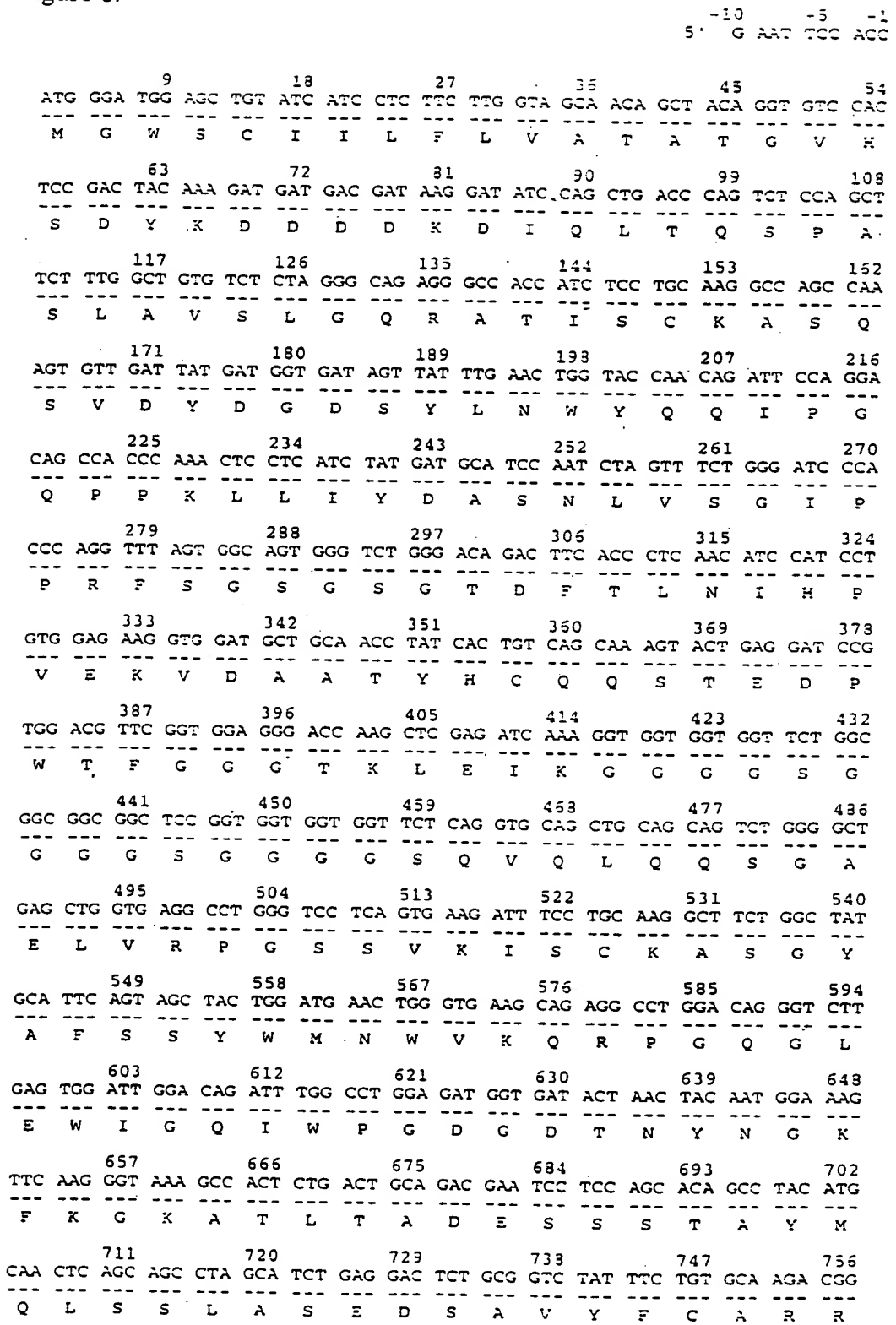

Cytotoxicity of bscCD19xCD3 in a 51-Cr release assay
with unstimulated PBMCs in different E:T ratios
E:T 20:1
E:T 10:1
E:T 5:1
E:T 2,5:1
specific lysis (%)
0
5
10
15
20
25
30
35
40
45
50
100
10
1
0,1
0,01
0,001
bscCD19xCD3 concentration
(ng/ml)
HD37xOKT3
bsc17-1AxCD3
medium

Figure 8: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S D Y K D D D D K D I Q L T Q S P A
117 126 135 144 153 162
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S L A V S L G Q R A T I S C K A S Q
171 180 189 198 207 216
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CAG CCA CCC AAA CTC CTC ATC TAT GAT GCA TCC AAT CTA GTT TCT GGG ATC CCA
Q P P K L L I Y D A S N L V S G I P
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E L V R P G S S V K I S C K A S G Y
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657 666 675 684 693 702
TTC AAG GGT AAA GCC ACT CTG ACT GCA GAC GAA TCC TCC AGC ACA GCC TAC ATG
F K G K A T L T A D E S S S T A Y M
711 720 729 738 747 756
CAA CTC AGC AGC CTA GCA TCT GAG GAC TCT GCG GTC TAT TTC TGT GCA AGA CGG
Q L S S L A S E D S A V Y F C A R R

Figure 8 (continued)

```
          765        774        783        792        801        810
GAG ACT ACG ACG GTA GGC CGT TAT TAC TAT GCT ATG GAC TAC TGG GGC CAA GGG
---------- ---------- ---------- ---------- ---------- ----------
 E   T   T   T   V   G   R   Y   Y   Y   A   M   D   Y   W   G   Q   G

          819        828        837        846        855        864
ACC ACG GTC ACC GTC TCC TCC GGA GGT GGT GGA TCC GAT ATC AAA CTG CAG CAG
---------- ---------- ---------- ---------- ---------- ----------
 T   T   V   T   V   S   S   G   G   G   G   S   D   I   K   L   Q   Q

          873        882        891        900        909        918
TCA GGG GCT GAA CTG GCA AGA CCT GGG GCC TCA GTG AAG ATG TCC TGC AAG ACT
---------- ---------- ---------- ---------- ---------- ----------
 S   G   A   E   L   A   R   P   G   A   S   V   K   M   S   C   K   T

          927        936        945        954        963        972
TCT GGC TAC ACC TTT ACT AGG TAC ACG ATG CAC TGG GTA AAA CAG AGG CCT GGA
---------- ---------- ---------- ---------- ---------- ----------
 S   G   Y   T   F   T   R   Y   T   M   H   W   V   K   Q   R   P   G

          981        990        999       1008       1017       1026
CAG GGT CTG GAA TGG ATT GGA TAC ATT AAT CCT AGC CGT GGT TAT ACT AAT TAC
---------- ---------- ---------- ---------- ---------- ----------
 Q   G   L   E   W   I   G   Y   I   N   P   S   R   G   Y   T   N   Y

         1035       1044       1053       1062       1071       1080
AAT CAG AAG TTC AAG GAC AAG GCC ACA TTG ACT ACA GAC AAA TCC TCC AGC ACA
---------- ---------- ---------- ---------- ---------- ----------
 N   Q   K   F   K   D   K   A   T   L   T   T   D   K   S   S   S   T

         1089       1098       1107       1116       1125       1134
GCC TAC ATG CAA CTG AGC AGC CTG ACA TCT GAG GAC TCT GCA GTC TAT TAC TGT
---------- ---------- ---------- ---------- ---------- ----------
 A   Y   M   Q   L   S   S   L   T   S   E   D   S   A   V   Y   Y   C

         1143       1152       1161       1170       1179       1188
GCA AGA TAT TAT GAT GAT CAT TAC TGC CTT GAC TAC TGG GGC CAA GGC ACC ACT
---------- ---------- ---------- ---------- ---------- ----------
 A   R   Y   Y   D   D   H   Y   C   L   D   Y   W   G   Q   G   T   T

         1197       1206       1215       1224       1233       1242
CTC ACA GTC TCC TCA GTC GAA GGT GGA AGT GGA GGT TCT GGT GGA AGT GGA GGT
---------- ---------- ---------- ---------- ---------- ----------
 L   T   V   S   S   V   E   G   G   S   G   G   S   G   G   S   G   G

         1251       1260       1269       1278       1287       1296
TCA GGT GGA GTC GAC GAC ATT CAG CTG ACC CAG TCT CCA GCA ATC ATG TCT GCA
---------- ---------- ---------- ---------- ---------- ----------
 S   G   G   V   D   D   I   Q   L   T   Q   S   P   A   I   M   S   A

         1305       1314       1323       1332       1341       1350
TCT CCA GGG GAG AAG GTC ACC ATG ACC TGC AGA GCC AGT TCA AGT GTA AGT TAC
---------- ---------- ---------- ---------- ---------- ----------
 S   P   G   E   K   V   T   M   T   C   R   A   S   S   S   V   S   Y

         1359       1368       1377       1386       1395       1404
ATG AAC TGG TAC CAG CAG AAG TCA GGC ACC TCC CCC AAA AGA TGG ATT TAT GAC
---------- ---------- ---------- ---------- ---------- ----------
 M   N   W   Y   Q   Q   K   S   G   T   S   P   K   R   W   I   Y   D

         1413       1422       1431       1440       1449       1458
ACA TCC AAA GTG GCT TCT GGA GTC CCT TAT CGC TTC AGT GGC AGT GGG TCT GGG
---------- ---------- ---------- ---------- ---------- ----------
 T   S   K   V   A   S   G   V   P   Y   R   F   S   G   S   G   S   G

         1467       1476       1485       1494       1503       1512
ACC TCA TAC TCT CTC ACA ATC AGC AGC ATG GAG GCT GAA GAT GCT GCC ACT TAT
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 T   S   Y   S   L   T   I   S   S   M   E   A   E   D   A   A   T   Y

         1521       1530       1539       1548       1557       1566
TAC TGC CAA CAG TGG AGT AGT AAC CCG CTC ACG TTC GGT GCT GGG ACC AAG CTG
---------- ---------- ---------- ---------- ---------- ----------
 Y   C   Q   Q   W   S   S   N   P   L   T   F   G   A   G   T   K   L

         1575       1584       1593
GAG CTG AAA CAT CAT CAC CAT CAT CAT TAG TCG AC 3'
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 E   L   K   H   H   H   H   H   H   *
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